

EXHIBIT 29

5'-Terminal Cap Structure in Eucaryotic Messenger Ribonucleic Acids

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INTRODUCTION

Over the past 10 years the study of the structure, function, and biosynthesis of messenger ribonucleic acid (mRNA) in eucaryotic cells has been an area of intense biochemical research in various laboratories. Gratifyingly, these studies have produced fascinating discoveries which have enabled us to approach an understanding of the regulation of eucaryotic gene expression. Unlike procaryotic mRNA's, eucaryotic mRNA's are synthesized in the nucleus of a cell as part of a long transcriptional precursor unit (commonly referred to as heterogeneous nuclear RNA [hnRNA]). These precursors are apparently subjected to various post-transcriptional modifications and tailoring before mature mRNA's are finally transported to the cyto-

plasm (48). These post-transcriptional processes include cleavage, 5'-terminal capping, methylation, 3'-terminal polyadenylation, and splicing. Although the mRNA's in the cytoplasm carrying the hallmarks of these modifications can be studied easily, the precise molecular events which occur within the nucleus and lead to these modifications are not yet clearly understood. The main stumbling blocks to such investigations have been the difficulties in radiolabeling RNAs within nuclei in satisfactory amounts, characterizing the enzymes involved in the modification processes, and finally developing a well-defined isolated nuclear system for the in vitro study of transcription and RNA modification. Recently, great progress has been made in this direction because viruses have been used as tools in the study of cellular mRNA biosynthesis. Many vi-

ruses contain virion-associated RNA polymerases which synthesize mRNA's in vitro that carry modifications identical to those of cellular mRNA's (26). In addition, many deoxyribonucleic acid (DNA)-containing viruses replicate in cell nuclei, and in transformed cells their genomes become part of the cellular material by integration (57). Virus-specific mRNA's are synthesized in the nucleus, processed, modified, and transported to the cytoplasm, presumably by the same mechanism as cellular mRNA's. Thus, the recent understanding of mRNA structure and function has been facilitated by the continuing search into the processes involved in viral mRNA biosynthesis.

This review deals primarily with only one of the important modifications of eucaryotic mRNA's, namely 5'-terminal capping (211). The majority of eucaryotic mRNA's have been found to contain a novel 5'-terminal structure. In this 5'-cap a guanosine residue blocks the 5'-terminal penultimate base of the mRNA through a unique 5'-5' linkage containing three phosphate groups (Fig. 1). The blocking guanosine residue universally contains a methyl group at the N-7 position, and the 5'-terminal penultimate base and its adjoining residue are also sometimes methylated in the ribose moiety or when the penultimate residue is an adenosine in the N-6 position as well. The unique 5'-terminal cap structure of eucaryotic mRNA's clearly differentiates them from the polycistronic procaryotic mRNA's, which contain unblocked triphosphate ends (7). These observations also indicate that the mode of mRNA biosynthesis in the eucaryotic system is more complex than in the procaryotic system; this is commensurate with the increased complexity of the genetic organization of eucaryotic species.

Since the discovery of the cap structure in

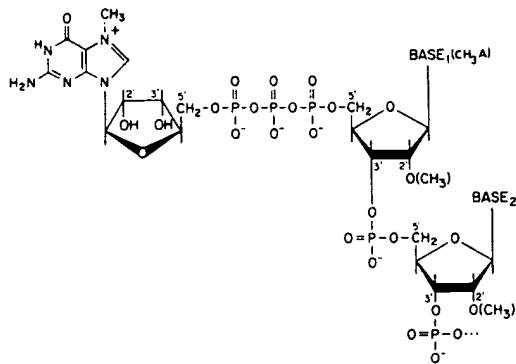


FIG. 1. Structure of 5' cap. When base₁ is adenosine, it is methylated at the N-6 position.

1974, a great deal of work has been done to elucidate its structure, synthesis, and functional role. I have attempted to review as much as possible from both the published and the unpublished data available to me, highlighting the significant findings rather than describing in detail all of the papers published to date. Clearly, there are omissions, for which the responsibility is mine. The excellent reviews by Shatkin (224) and Filipowicz (70) have dealt specifically with the cap structures, and the reviews by Rottman (210), Adams (7), and Busch et al. (34) have dealt with these structures in their summaries on eucaryotic mRNA structure and function.

5'-TERMINAL CAP STRUCTURE IN MESSENGER RIBONUCLEIC ACID (mRNA)

Discovery

The existence of a 5'-terminal blocked structure at the 5' terminus of an RNA molecule was first shown by Reddy et al. (196). These researchers studied the low-molecular-weight nuclear RNAs (4 to 8S) derived from Novikoff hepatoma cells. The primary sequence analysis of one of the RNA species revealed a blocked 5' terminus with a hypermethylated guanosine residue (2,2,7-trimethylguanosine) linked to a 2'-*O*-methylated adenosine residue through a 5'-5' pyrophosphate (PP_i) linkage. Since the precise functions of the small nuclear RNA species were not clear, the importance of the cap structure in these RNAs was not immediately evident. The 5'-terminal structures of eucaryotic mRNA's were believed to be different from those of pro-caryotic mRNA's since they were resistant to phosphorylation by polynucleotide kinase after prior treatment with alkaline phosphatase (211), a property shared with the low-molecular-weight nuclear RNAs. The discovery of the cap structure in mRNA's was achieved through a series of original observations made by various researchers. Perry and Kelly (186) reported the existence of methylated mRNA's in mouse L-cells. They reported the presence of approximately 2.2 methyl groups per 1,000 nucleosides in polyadenylic acid [poly(A)]-containing L-cell mRNA. In addition, a portion of the methyl label was found to be present in alkali-resistant oligonucleotides, suggesting that some of the methyl groups may be located in the ribose moieties in adjacent nucleotides or at the 5' termini of the mRNA molecules. Similarly, the existence of methylated mRNA's in Novikoff hepatoma cells was reported by Desrosiers et al. (52). Miura et al. (161, 162) observed that the 5'

termini of the double-stranded genome RNAs of cytoplasmic polyhedrosis virus (CPV) and reovirus were scarcely labeled at their 5' termini with [γ - 32 P]adenosine triphosphate (ATP) by the polynucleotide kinase procedure. However, after sequential oxidation, β -elimination, and phosphomonoesterase digestion, both of the 5' ends of CPV and reovirus were successfully labeled with 32 P. These authors also showed that one of the strands of each of the 10 double-stranded genome RNAs of reovirus appeared to contain blocked 5' termini containing 2'-O-methylated guanosine (G^m). Furuichi (75), also working with CPV, showed that the virion-associated RNA polymerase requires S-adenosylmethionine (AdoMet) for transcription of the genome RNA in vitro. Moreover, all of the mRNA species synthesized in vitro were methylated as part of the initiation step of transcription, indicating that the purified virions of CPV contain a methyltransferase activity that transfers the methyl group of AdoMet to mRNA probably at or near the 5' end of nascent transcripts. Similar methyltransferase activities were later shown to be present in purified reovirus (68, 223), vaccinia virus (263), and vesicular stomatitis virus (VSV) (198). The actual identification of the site of methylation in mRNA and the eventual elucidation of the 5'-terminal methylated capped structure shown in Fig. 1 were reported independently by Furuichi et al. (79), Wei and Moss (264), and Furuichi and Miura (78), who used reovirus, vaccinia virus, and CPV, respectively. These results were followed quickly by a series of reports which demonstrated the presence of the cap structure in viral and eucaryotic mRNA's; these included reports on vaccinia virus by Urushibara et al. (256), VSV by Abraham et al. (2), mouse L-cells by Perry et al. (188, 189), rat hepatoma cells by Desrosiers et al. (53), HeLa cells by Furuichi et al. (81) and Wei et al. (261, 262), and mouse myeloma cells by Adams and Cory (8).

Isolation and Characterization of the Cap Structures

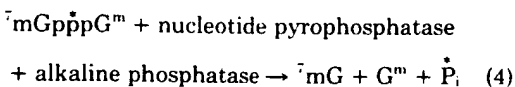
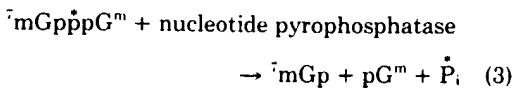
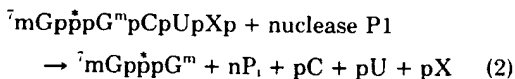
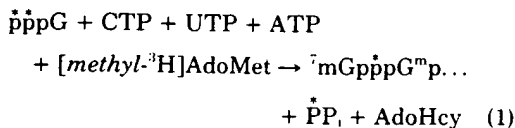
Viral mRNA's. The detailed method of isolation and characterization of the cap structures in mRNA's was established through studies of methylated mRNA's synthesized in vitro by the virion-associated polymerases of reovirus, vaccinia virus, CPV, and VSV (2, 78, 79, 264). The essential steps involved in the isolation and subsequent characterization of the cap structure, as exemplified for reovirus mRNA's (79), are described below.

(i) Phosphatase-resistant 5'-terminal

phosphates in methylated mRNA's. Under normal in vitro transcription conditions (i.e., in the absence of AdoMet) in the presence of [β - γ - 32 P]guanosine triphosphate (GTP), the reovirus mRNA's terminate with 5' ppGpC (83) (an asterisk denotes a labeled phosphate). However, in the presence of AdoMet, it was observed that 54% of the 5'-terminal labeled phosphate from the initiating nucleotide GTP was resistant to removal from the RNA by treatment with alkaline phosphatase. These results indicated that a population of the mRNA molecules synthesized in the presence of AdoMet must contain 5'-terminal ppGpC in a blocked structure.

(ii) Structure of the blocked 5' termini.

To study the nature of the phosphate-protecting group, mRNA synthesized in vitro in the presence of [β - γ - 32 P]GTP and [*methyl*- 3 H]AdoMet was digested with nuclease P1, an enzyme that cleaves phosphodiester linkages in nucleic acids to yield 5'-mononucleotides (74). All of the *methyl*- 3 H- and 32 P-labeled material migrated together between pA and pG markers on paper electrophoresis. The molar ratio of 32 P to *methyl*- 3 H was 1:2. The electrophoretic migration and the net negative charge (-3) of this material were not altered after digestion with alkaline phosphatase, indicating that the phosphate moiety was resistant to the enzyme. However, when the phosphatase-resistant structure was treated with a combination of alkaline phosphatase and nucleotide pyrophosphatase, all of the 32 P was converted to inorganic orthophosphate (P_i) and the 3 H radioactivity was divided equally between material that migrated with the authentic markers 7-methylguanosine (7 mG) and G^m . From these results, together with other confirmatory data, it appeared that the 5'-terminal blocked structure of reovirus mRNA's in vitro was 7 mGpppG m p.... The sequence of the reactions which are described above and led to the elucidation of the structure is as follows:



where CTP is cytidine triphosphate, UTP is uridine triphosphate, and AdoHcy is *S*-adenosyl-L-homocysteine.

(iii) **5'-5' Inverted linkage of the terminal nucleotides.** It is apparent from reaction 3, in which the products of nucleotide pyrophosphatase treatment of the cap structure are 5' ⁷mGp and pG, that a 5'-5' inverted linkage exists between the blocking guanosine base and the penultimate residue in the RNA. A nucleotide linkage of this type contains a 2',3'-*cis*-diol similar to the 3'-terminal nucleotides of RNA.

Cellular mRNA's. Isolation and characterization of the cap structures of cellular mRNA's have been carried out essentially by the following two methods. (i) The RNAs of cells were labeled uniformly *in vivo* with ³²P, and purified from the cytoplasmic fraction. The poly(A)-containing mRNA's were purified by repeated chromatography on oligodeoxythymidylic acid-cellulose columns (186). The purified mRNA's were then digested with ribonuclease (RNase) T₂, and the products were fractionated by electrophoresis on cellulose acetate at pH 3.5 and then on diethylaminoethyl paper at the same pH (8). A series of capped and methylated oligomers were obtained; these were further characterized chemically and enzymatically as described above. Using this method, Cory and Adams (45) identified a minimum of 27 oligonucleotides containing a capped structure in mRNA's isolated from mouse myeloma cells. (ii) The cap structures in mRNA's were labeled with methyl-³H by exposing exponentially growing cells to [methyl-³H]-methionine in medium containing a reduced concentration of methionine and supplemented with sodium formate, adenosine, and guanosine to prevent incorporation of methionine methyl groups via the one-carbon pool (186). The poly(A)-containing mRNA's were analyzed for cap structures by various enzymatic procedures, as described above. A similar approach was taken to analyze cap structures in mRNA's isolated from virus-infected cells (172). mRNA's were purified by chromatography on oligo-deoxythymidylic acid-cellulose or, in certain cases, by hybridization to viral genome RNA or deoxyribonucleic acid (DNA) (138).

The cap structures isolated from eucaryotic mRNA's and subsequently characterized have the general structure ⁷mG(5')ppp(5')N₁^mpN₂^mpN₃p..., where N is a purine or a pyrimidine. ⁷mG is universally present as the capping base, and the 5'-penultimate nucleoside (N₁) and its adjacent nucleoside (N₂) can also be methylated. The methylation of the latter two nucleosides may vary, generating the following types of

structures: ⁷mG(5')ppp(5')N₁pN₂p... (cap 0), ⁷mG(5')ppp(5')N₁^mpN₂p... (cap 1), and ⁷mG(5')ppp(5')N₁^mpN₂^mp... (cap 2). Various RNases have different modes of action on the cap-containing regions of the mRNA's. RNase T₂, a nonspecific RNase, does not cleave the phosphodiester bond adjacent to a 2'-*O*-methylated nucleoside. Thus, after RNase T₂ digestion, mRNA's containing cap 0, cap 1, and cap 2 yield ⁷mGpppN₁p, ⁷mGpppN₁^mpN₂p, and ⁷mGpppN₁^mpN₂^mpN₃p, respectively, in addition to the internal mononucleotides. Similar products are also generated by alkaline hydrolysis of mRNA's containing the corresponding cap species. Nuclease P1, on the other hand, generates the structures ⁷mG(5')ppp(5')N₁ (referred to as the cap core) from cap 0-containing mRNA's and ⁷mG(5')ppp(5')N₁^m from both cap 1- and cap 2-containing mRNA's. RNase A and RNase T₁ digestions of mRNA's generate cap-containing oligonucleotides terminated with 3'-pyrimidine and guanosine, respectively. Thus, by using various RNases, different cap-containing oligonucleotides of mRNA's can be isolated (for example, by binding of the oligonucleotides to dihydroxyborate cellulose [85a, 197]), and they can be further characterized by electrophoresis (45, 79), chromatography on diethylaminoethyl-cellulose (2, 264), or high-pressure liquid chromatography (52).

Properties of Cap Structures

The cap structures have some interesting, characteristic features that impart special properties to the 5'-terminal structures and thus to the mRNA's. These are described below.

(i) The capping base is universally ⁷mG, except for Sindbis virus mRNA, which contains a low level of ^{2,2,7}m₃G at the 5' terminus (see below). So far, no other terminal base besides ⁷mG has been found in the cap structure of eucaryotic mRNA's. Although ⁷mG is present internally in transfer RNA, it is absent in ribosomal RNA, hnRNA, and cytoplasmic mRNA. In the latter two RNA species ⁷mG is present only as part of the 5'-terminal cap. It is interesting to note that the N-7 atom of guanine is more susceptible to the action of alkylating compounds, and the conversion of guanine to 7-alkylguanine in nucleic acid is considered to be an important event underlying the mutagenic, cytotoxic, carcinogenic, and carcinostatic activities of biological alkylating agents (139). Alkylation of the N-7 atom of guanine in a nucleic acid also introduces a positive charge in the imidazole ring, which has been implicated in erroneous base pairing

with thymine in DNA (140). Moreover, alkylation of the N-7 atom facilitates rapid exchange of the H-8 atom with water, and the imidazole ring undergoes fission when treated with alkali (107). The 9-monosubstituted guanine derivatives, however, are resistant even when treated with hot strong alkali (107). Thus, alkylating of the N-7 atom in guanine imparts some special chemical properties to the capped 5' termini of mRNA's, but the precise functions of these are not clear at the present time.

(ii) The unique 5'-5' phosphate linkage in the cap structure results in the retention of a 2',3'-*cis*-diol on the 5'-terminal ⁷mG. In this respect, the 3' and 5' ends of the mRNA appear to be identical. The presence of the *cis*-diol at the 5' end permits special chemical reactions usually restricted to the 3' end, such as periodate oxidation followed by labeling with [³H]borohydride (173). The capping base may also be selectively removed by β -elimination by treating the oxidized nucleoside with aniline (173). The reactive dialdehyde may be reduced with cyanoborohydride in the presence of protein, yielding covalently linked protein-RNA conjugates (234). It is also possible to isolate 5'-terminal fragments of mRNA by using affinity chromatography on resins containing covalently bound borate, which were originally developed for selecting 3'-terminal fragments of RNA (208a).

(iii) Nuclear magnetic resonance studies (54, 126, 127) of the capped structure ⁷mGpppA^mpA have revealed that the three bases form a closely stacked array, resulting in increased stability. It appears that ⁷mG5'p has enough flexibility so that it can intercalate between the two adenine residues. The 2'-O-methyl group present in the penultimate nucleoside induces sufficient conformational strain that the backbone of the A^mpA segment assumes an extended spatial configuration, enabling the ⁷mG5'p residue to intercalate between the adenine bases (111). The presence of a long triphosphate bridge would allow ⁷mG5'p to bend and intercalate between the adenines. Thus, it appears that the overall spatial configuration of the mRNA terminus ⁷mG5'ppp5'A^mpA is a self-intercalating one which imparts a unique feature to the terminus. These features may affect stability, allow interaction with specialized proteins in ribosomes, etc.

Distribution of Capped mRNA's in Nature

After the discovery of cap structures in reovirus, vaccinia virus, CPV, and VSV, a search was made for such structures in a variety of other viral and cellular mRNA's. As Tables 1

and 2 show, cap structures are present almost universally in eucaryotic mRNA's, with only a few exceptions. Capped mRNA's are present in yeasts, molds, plants, insects, and higher organisms. The only reported exceptions among cellular mRNA's are the poly(A)-containing mRNA's isolated from HeLa cell mitochondria (90, 248). These mRNA's may contain ppA at the 5' termini. The 5' termini of maternal mRNA's obtained from developing oocytes of the tobacco hornworm (*Manduca sexta*) apparently contain a cap structure but lack methylation of the capping guanosine (117). In plant and animal viruses almost all mRNA's of both the DNA- and RNA-containing viruses contain a cap structure, except picornaviruses (108, 142) and three plant viruses, satellite tobacco necrosis virus (144, 269) and its helper virus (143) and cowpea mosaic virus (CMV) (50, 241). In the case of picornaviruses, including poliovirus and encephalomyocarditis virus, it has been shown that a protein (molecular weight, 12,000) is connected covalently to the 5'-terminal uracil of the viral genome through a phosphodiester linkage to a tyrosine residue in the protein (88, 108). A similar protein-linked genome has also been described for CMV (50, 241). Satellite tobacco necrosis virus, however, contains a di- or triphosphate (p)ppA at the 5' end (144), which is similar to bacterial and bacteriophage mRNA's (144, 269).

In a survey of cap structures present in eucaryotic mRNA's (Tables 1 and 2), an interesting pattern emerges with regard to the cap composition of various types of mRNA's. These features can be summarized as follows.

Viral mRNA's. All mRNA's contain ⁷mG as the capping base except the few viral mRNA's that do not contain a cap (Table 1). Only the mRNA strand (i.e., the positive strand) is capped, whereas the antisense strand, such as the genome of VSV and influenza virus and one strand of the duplex genome RNA of reovirus and CPV, usually has a di- or triphosphate 5' end rather than a cap. This indicates that capping is probably restricted to mRNA's. The exceptions are the low-molecular-weight nuclear RNAs (46, 196, 202) which are capped but do not act as messengers in *in vitro* protein synthesizing systems (195). The 5'-penultimate base of viral mRNA's is always a purine, which may or may not be methylated. This may indicate that in viruses initiation of RNA synthesis or processing of RNA may be purine specific. When the 5'-penultimate base is adenosine, it exists in the majority of cases in a dimethylated form, N⁶-methyl-2'-O-methyladenosine (N⁶mA^m) (260). Plant viruses usually contain a cap 0 structure,

TABLE 1. 5'-Terminal cap structures in viral mRNA's

Host and virus ^a	Type of genome	Site of replication	5'-Terminal structure			Internal methylation	Reference(s)
			Virion RNA	In vitro mRNA	Cytoplasmic mRNA		
Mammal							
Poliovirus	ssRNA(+) (+)	Cytoplasm	Protein-pUp		pUp	None	108, 142
EMC	ssRNA(+) (+)	Cytoplasm	Protein-pNp		pNp	None	88
FMDV	ssRNA(+) (+)	Cytoplasm	Protein-pNp		pNp	None	216
Sindbis virus	ssRNA(+) (+)	Cytoplasm	mGpppApUpYpGp		mGpppApUpGp	m ¹ C	59-62, 103, 112
Calicivirus	ssRNA(+) (+)	Cytoplasm	Protein-linked		No cap	NID ^d	27, 33, 64
Dengue virus	ssRNA(+) (+)	Cytoplasm	mGpppA ^m pNp		mGpppA ^m p	None	42
VSV	ssRNA(-) (-)	Cytoplasm	(p)ppA		mGpppA ^m p	None	2, 102, 171, 204, 254
Influenza virus	ssRNA(-) (-)	Nucleus(?)	pppA		mGpppA ^m p/G ^m p	3 m ¹ A	134, 169, 193
Newcastle disease virus	ssRNA(-) (-)	Cytoplasm	NID		mGpppNp ⁻	None	43, 44
Reovirus	dsRNA(±)	Cytoplasm	mGpppG ^m (+)		mGpppG ^m pC ^m p	None	53a, 67, 79, 82
Rous sarcoma virus	ssRNA(+) (+)	Nucleus	ppG(-)		NID	10-12 m ¹ A	24, 118
Avian sarcoma virus	ssRNA(+) (+)	Nucleus	mGpppG ^m pCp		NID	10 m ¹ A	86, 243
Moloney murine leukemia virus	ssRNA(+) (+)	Nucleus	mGpppG ^m pCp		NID	15-23 m ¹ A	205
Feline leukemia virus	ssRNA(+) (+)	Nucleus	No cap		mGpppG ^m pAp	10 m ¹ A	252, 253
Insect							
CPV	dsRNA(±)		mGpppA ^m p(+) ppA(-)		NID	None	78
Fish							
Spring viremia of carp	ssRNA(-) (-)	Cytoplasm	pppA		mGpppA ^m p	ND	96, 212
Plant							
Wound tumor virus	dsRNA(+) (+)		NID		NID	ND	199
Brome mosaic virus	ssRNA(+) (+)		mGpppGp		NID	None	49
Tobacco mosaic virus	ssRNA(+) (+)		mGpppGp		NID	None	119, 279
Alfalfa mosaic virus	ssRNA(+) (+)		mGpppGp		NID	None	191
Turnip yellows virus	ssRNA(+) (+)		mGpppGp/A		NID	None	95, 128
Cucumber mosaic virus	ssRNA(+) (+)		mGpppNp		NID	None	246
Cucumber mosaic virus-associated RNA (CARNA 5)			mGpppGpU		NID	None	200
Barley stripe mosaic virus	ssRNA(+) (+)		mGpppN		NID	None	9
Potato virus X	ssRNA(+) (+)		mGpppGpA		NID	None	235
Tobacco necrotic virus	ssRNA(+) (+)		ppApG		NID	None	143
Satellite tobacco necrotic virus	ssRNA(+) (+)		ppApG		NID	None	144, 269

TABLE 1.—Continued

Host and virus ^a	Type of genome ^b	Site of replication	5'-Terminal structure			Internal methylation	References ^c
			Virion RNA	In vitro mRNA	Cytoplasmic mRNA		
CMV	ssRNA(+)		No cap, protein-linked		ND	ND	129, 241
Fungus <i>Penicillium chrysogenum</i>	dsRNA(±)		mGpppN ^m p(+) ppAGY(-)		ND	ND	249
Mammal Vaccinia virus	DNA	Cytoplasm		mGpppA ^m p/G ^m p	mGpppm'A ^m p/G ^m p	None	260, 264
Adenovirus	DNA	Nucleus			mGpppm'A ^m p/A ^m pN ₁ ^m p/	m'A, m'C	99, 100, 170, 273
Simian virus 40	DNA	Nucleus			mGpppm'A ^m p/G ^m p	m'A	10, 36, 91, 138
Herpes simplex virus type 1	DNA	Nucleus			mGpppN ₁ ^m pN ₂ ^m p ^d	m'A	166
Polyoma virus	DNA	Nucleus			mGpppm'A ^m p/G ^m pN ₂ ^m p	m'A	72

^a Abbreviations: EMC, encephalomyocarditis virus; FMDV, foot and mouth disease virus.^b ss, Single stranded; ds, double stranded.^c mRNA contains a small proportion of m₂²⁷G and m₁^{2,27}G at the 5' terminus (112).^d ND, Not determined.^e H. O. Stone, personal communication.^f Some of the early mRNA's contain cap 1 with N₁ = methyluridine (see reference 100).^g N₁ = m⁶A^m, methyladenosine, or methylguanosine.

TABLE 2. 5'-Terminal cap structures in cellular mRNA's

Cells	Cap structure ^a			Internal methylation	Reference(s)
	Cap 0	Cap 1	Cap 2		
Human HeLa cells	None	N ₁ = Pu ^m , Py ^m , m ^a A ^m	N ₂ = Pu ^m , Py ^m	m ^a A	81, 261, 262, 266
HeLa cell histone	None	N ₁ = A ^m , m ^a A ^m , G ^m	N ₂ = Pu ^m , Py ^m	None	167, 242
Mouse myeloma	None	N ₁ = Pu ^m , Py ^m , m ^a A ^m	N ₂ = Pu ^m , Py ^m	m ^a A	8, 45
Mouse erythroid	None	N ₁ = Pu ^m , Py ^m , m ^a A ^m	N ₂ = Pu ^m , Py ^m	m ^a A	101
Mouse L-cells	None	N ₁ = Pu ^m , Py ^m , m ^a A ^m	N ₂ = Pu ^m , Py ^m	m ^a A	188
Mouse kidney	None	N ₁ = N ^m	N ₂ = N ^m	Present	182, 183
Rat hepatoma	None	N ₁ = Pu ^m , Py ^m , m ^a A ^m	N ₂ = Pu ^m , Py ^m	m ^a A	52, 53
Hamster kidney (BHK-21)	None	N ₁ = Pu ^m , Py ^m , m ^a A ^m	N ₂ = Pu ^m , Py ^m	m ^a A, m ^a C	61, 172
Monkey kidney (BSC-1)	None	N ₁ = Pu ^m , Py ^m , m ^a A ^m	N ₂ = Pu ^m , Py ^m	m ^a A	138
Mouse immunoglobulin	None	N ₁ = G ^m , m ^a A ^m	N ₂ = A ^m	m ^a A	47, 156
Human globin	None	N ₁ = m ^a A ^m , A ^m	N ₂ = C ^m	None	40
Mouse globin	None	N ₁ = m ^a A ^m , A ^m	N ₂ = C ^m	None	39
Rabbit globin	None	N ₁ = m ^a A ^m	N ₂ = C ^m	None	47, 148, 150
Duck globin	None	N ₁ = N ^m	ND ^b	None	190
Ovalbumin	N ₁ = A	N ₁ = m ^a A ^m , A ^m	N ₂ = Py ^m	ND	154
Protamine		Contains cap			86
<i>Drosophila</i>	N ₁ = C	N ₁ = Py ^m > Pu ^m	N ₂ = Pu ^m , Py ^m	ND	147
Silk fibroin	None	N ₁ = A ^m	N ₂ = U ^m	m ^a A	274
<i>Aedes albopictus</i>	None	N ₁ = Pu ^m , Py ^m	N ₂ = Pu ^m , Py ^m	m ^a A	113
Tobacco hornworm oocyte	GpppN				117
Brine shrimp	N ₁	ND	ND	ND	174
Sea urchin embryo	None	N ₁ = Pu ^m > Py ^m	None	m ^a A	69, 244
Slime mold	N = A > G	N ₁ = A ^m (10%)	None	None	58
<i>Neurospora</i>	N = A > G	None	None	None	220
Yeast	N = A > G	None	None	None	51, 152, 240
Wheat embryo	N ₁	None	None	None	98, 125, 213
Maize	N ₁	None	None	None	178
Soybean seeds	N ₁	N ₁ = N ^m	None	None	247

^a Cap 0 denotes 7mGpppN₁pN₂p...; cap 1 denotes 7mGpppN₁^mpN₂p...; and cap 2 denotes 7mGpppN₁^mpN₂^mp...
N₁ and N₂ are purine (Pu) or pyrimidine (Py) ribonucleosides.

^b ND, Not determined.

but among animal viruses cap 0 structures are rare, with the exception of Sindbis virus genome RNA (103) and Newcastle disease virus mRNA synthesized in vitro (44). The majority of viral mRNA's contain a cap 1 (in vitro mRNA's) or cap 2 (in vivo mRNA's) structure. In addition to methyl groups in the cap structure at the 5' terminus of an mRNA, N^a-methyladenosine (N^amA) is often present internally in a variety of mRNA's. Viruses that replicate in the cytoplasm of infected cells (such as VSV, reovirus, and vaccinia virus) do not contain internal N^amA residues, whereas those replicating in the nucleus (simian virus 40, adenovirus, etc.) contain N^amA located internally. The exception is Sindbis virus, in which N^amA is present in in vivo mRNA's but not in genome RNA. These results may indicate that the methylation of internal adenosines in mRNA is a nuclear event. 5-Methylcytidine (m⁵C) also has been shown to be present internally in adenovirus (233) and Sindbis virus (59) mRNA's. In the latter case, m⁵C represents the only internal methylated base. Interestingly, a fraction of the Sindbis virus

mRNA (26S) molecules from infected cells contain, in addition to the usual 7mG, the hypermodified congeners m₂^{2,7}G, and m₃^{2,2,7}G (112), similar to the low-molecular-weight nuclear RNAs (46, 202).

Cellular mRNA's. A variety of 5'-terminal capped sequences were found in higher eucaryotic mRNA's, including those of HeLa cells, myeloma cells, baby hamster kidney cells, etc. In the well-studied mouse myeloma cells (MPC-11-66) (45), a minimum of 27 5'-terminal sequences were reported. Unlike viral mRNA's, the 5'-penultimate base can be any of the four standard bases or N^amA^m. The second base can also be any one of the standard four. mRNA's containing 7mG(5')ppp(5')C^mpUp appear to be present in relatively high abundance (19%), compared with other sequences, which occur at levels of less than 1%. It is not known whether the different termini are associated with specific classes of mRNA or have any functional significance. In this respect, cellular mRNA's are more complex than viral mRNA's. The presence of pyrimidines in the 5'-penultimate position in

cellular mRNA's suggests the possibility that they may not be primary transcripts, assuming that all RNA chains are initiated by purines (153). On the other hand, it is quite possible that initiation may occur by pyrimidines (209). A better understanding of the precise nature of initiation in eucaryotic systems will help to clarify these observations.

The degrees of methylation in the cap structures are also different in cellular and viral mRNA's (Tables 1 and 2). Unlike viral mRNA's, cap 1 and cap 2 structures are present in all cellular mRNA's, although the cap 1 structure is about five times more abundant than the cap 2 structure (187). As in viral mRNA's, 5'-penultimate adenosine residues are predominantly N^6mA^m . The only exception is in *Drosophila melanogaster* mRNA's (147), which contain cap 0 structures in addition to cap 1 and cap 2 structures. Moreover, these mRNA's do not contain N^6mA^m . Like higher mammal mRNA's, *Drosophila* cell mRNA's contain purines and pyrimidines at the N_1 bases of the cap 1 structures. However, the majority of poly(A)-containing cytoplasmic RNA molecules from heat-shocked *Drosophila* cells contain purine almost exclusively as the N_1 base of cap 1. These results are consistent with other results, indicating that a special set of mRNA's is turned on during heat shock. It was also observed in *Drosophila* mRNA's that two RNase T₂-resistant dinucleotides were present in the fingerprint (147). These dinucleotides are not capped and may or may not be methylated; their precise compositions are not known. Interestingly, similar spots in the fingerprints of mouse myeloma cell mRNA's were also found (45), but they were not detected in sea urchin or *Dictyostelium* mRNA's (58).

As Table 2 shows, the diversity of cap structures is restricted to certain eucaryotes. A comparison of the 5'-terminal sequences of mammalian mRNA's and those of lower eucaryote mRNA's reveals phylogenetic trends in the cap structures. For example, only cap 0 is found in yeast mRNA and predominates in the mRNA of the unicellular slime mold *Dictyostelium discoideum*. Moreover, the N_1 base is a purine in both of these organisms. Studies of sea urchin mRNA have identified only cap 1 structures, with a purine in more than 80% of the N_1 bases. In metazoan cell mRNA (*Drosophila*) three varieties of caps are found, with a random frequency of purines and pyrimidines but no N^6mA^m in the N_1 base. In the fibroin mRNA of the silkworm and in higher organisms, cap 1 and cap 2 structures predominate, and N^6mA^m appears in the N_1 position. These observations indicate that the cap structure and its adjacent bases increase

in complexity in the more developed genome structures of higher eucaryotes. The precise function of these modified structures in relation to gene regulation is not known.

The internal N^6 -methylation of eucaryotic cellular mRNA's also has an interesting pattern. The mRNA's isolated from higher eucaryotic organisms, such as HeLa cells (human), L-cells (mouse), and BSC cells (monkey), invariably contain N^6mA internally. The striking exceptions are the globin and histone mRNA's, which do not contain this methylated base internally (167, 190). In contrast to the findings described above, the mRNA's isolated from lower eucaryotic organisms, such as slime molds, yeasts, etc., do not contain internal N^6mA . Although the reasons for these differences are not known, they may reflect some basic differences among eucaryotic organisms that may directly relate to the mode of mRNA biosynthesis. The methylation of internal adenosine appears to occur at specific sites in RNA. In HeLa cells and L-cells two methylated internal sequences were detected, namely, Gpm⁶ApC and Apm⁶ApC (217, 266). Interestingly, in avian sarcoma virus genome (55), Rous sarcoma virus (24), and simian virus 40 late mRNA's (36) identical sequences were found. Like some animal viruses, cultured hamster kidney cells and Novikoff mRNA's have been shown to contain internal m⁶C at levels of 20 and 3%, respectively, of the total, the remainder being N^6mA .

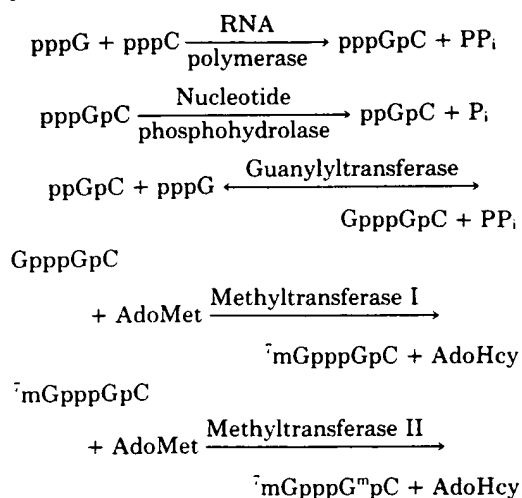
MECHANISM OF THE CAPPING REACTION

Viral mRNA's

Elucidation of the various steps involved in the formation of the cap structure was made possible through studies of animal viruses that carry virion-associated RNA polymerase. Under appropriate reaction conditions, mRNA's synthesized in vitro by virion-associated RNA polymerase may contain 5'-terminal uncapped structures, capped but not methylated structures, cap 0 structures, or cap 1 structures, demonstrating that these viruses contain all of the enzymes necessary for the synthesis of cap. The mechanisms of capping that have been well elucidated by studying reovirus (83), vaccinia virus (168), CPV (76), VSV (17), influenza virus (133), etc., are summarized below.

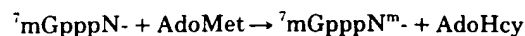
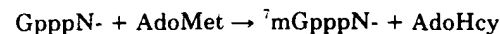
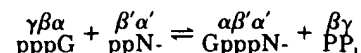
Reovirus. Virion-associated RNA polymerase transcribes in vitro the 10 double-stranded genome RNAs into 10 mRNA species containing predominantly the 5'-terminal sequence ppGpC... (224). In the presence of the methyl donor AdoMet, the predominant 5'-terminal structure is ⁷mGpppG^mpC... (79). If methylation

is prevented by AdoHcy, 5'-terminal ppGpC... predominates (79). Reovirus cores incubated in the presence of GTP, cytidine triphosphate, and AdoMet synthesize the cap structure $^7\text{mGpppG}^{\text{m}}\text{pC}$ (83). The dinucleotide ppGpC functions as a substrate for a core-associated guanylyltransferase and is converted to GpppGpC by the addition of pG from GTP. For optimal conversion, both a diphosphate terminus and a phosphodiester bond are required in the acceptor. pGpC is not a substrate, but pppGpC is utilized after removal of the γ -phosphate by a core-associated nucleotide phosphohydrolase. GpppGpC is hydrolyzed by the cores to ppGpC in the presence of PP_i ; however, this hydrolysis is markedly decreased by methylation at N-7 of guanosine. It was further shown that the β - γ -imido analog of GTP, GMPPNHP, can replace GTP for transcription; under these conditions, the 5'-terminal structure is pNHppGpC (84). Thus, hydrolysis of the β - γ bond of GTP in the acceptor RNA molecule is needed for cap formation. Based on these observations, the following reaction series was proposed for the synthesis of reovirus mRNA caps:



Thus, it appears that in reovirus mRNA's the α - and β -phosphates of GTP and the α -phosphate of the capping GTP are retained in the cap structure and that capping and initiation of RNA synthesis occur concurrently.

Vaccinia virus. mRNA's synthesized in vitro by vaccinia virus in the presence of AdoMet contain two 5'-terminal structures, $^7\text{mGpppG}^{\text{m}}\text{p}...$ and $^7\text{mGpppA}^{\text{m}}\text{p}...$ (264). The virion contains guanylyltransferase and methyltransferase activities, which modify the 5' ends of mRNA's. The mechanism of the capping reaction is similar to the reovirus system, and the following scheme has been established (168):



At limited concentrations of AdoMet, methylation occurs at the N-7 G position, whereas at higher concentrations ribose methylation of the penultimate N_1 base occurs. (See below for a detailed description of the properties of the vaccinia virus guanylyltransferase and methyltransferases which have been solubilized and purified from the virion in an active form.) Although the properties of the purified enzymes have been well characterized, it is still not clear whether the specificities of the enzymes within the virus core are the same as those of the highly purified enzymes.

Cytoplasmic polyhedrosis virus. Like reovirus, the RNA polymerase of CPV transcribes 10 genome segments in vitro (78). In contrast to reovirus and vaccinia virus, the synthesis of mRNA in vitro is stimulated considerably by the addition of AdoMet; the resulting mRNA contains the 5'-terminal capped structure $^7\text{mGpppA}^{\text{m}}\text{pGp}...$ (78). Moreover, the competitive inhibitor of methylation AdoHcy also effectively stimulates mRNA synthesis in vitro, resulting in the synthesis of viral mRNA containing 5'-terminal GpppA and ppA. Thus, it appears that CPV may have an additional regulatory mechanism(s) by which AdoMet and AdoHcy "switch on" mRNA synthesis, probably as allosteric effectors that influence the RNA polymerase and capping enzymes (85) (see below). It was further shown that the β , γ -imido analog of ATP (AMPPNHP) failed to support methylation and RNA synthesis, whereas similar analogs of GTP, cytidine triphosphate, and uridine triphosphate were effective substrates for RNA synthesis and methylation (76). These results were interpreted as due to the inability of AMPPNHP to replace 5'-ATP because it cannot be processed by phosphohydrolase, an enzyme involved in cap formation (see above). It was shown that during partial reactions in vitro with ATP and GTP as substrates, GpppA was produced and subsequently methylated to $^7\text{mGpppA}^{\text{m}}\text{p}$ in the presence of AdoMet (227). However, neither the 5'-capped compounds nor other dinucleotides, such as pppApG and ppApG, stimulated mRNA synthesis in vitro in the absence of AdoMet or AdoHcy (Y. Furuichi, personal communication). These results may imply that CPV RNA polymerase, for its full function, may require that some functional barrier be removed by an unknown process mediated by the presence of AdoMet or AdoHcy (see below).

Vesicular stomatitis virus. The virion-associated RNA polymerase of VSV synthesizes in vitro five mRNA species that are capped and have the 5' sequence GpppApApCpApG (3, 197). These 5' ends are quantitatively methylated to $^7\text{mGpppA}^{\text{m}}\text{pApCpApG}$... in the presence of AdoMet (2). The interesting features of the cap structures of VSV mRNA that distinguish them from the other viral mRNA cap structures described above are that (i) the α -phosphate of ATP and the α - and β -phosphates of GTP are incorporated into the cap structure (2), (ii) exogenously added ppA-containing mRNA's or GpppA cannot be capped or methylated, respectively, in the in vitro system (1), and (iii) the methyltransferase reaction takes place as follows (250):

GpppA- + AdoMet (<0.1 μM)

methyltransferase
→ GpppA $^{\text{m}}\text{p}$ -

GpppA $^{\text{m}}\text{p}$ - + AdoMet (>5 μM)

methyltransferase
→ $^7\text{mGpppA}^{\text{m}}\text{p}$ -

These reactions are different from those in the reovirus and vaccinia virus systems, in which 7-methylation precedes 2'-O-methylation even at low AdoMet concentrations. It appears that capping and methylation in the VSV system are tightly coupled to transcription (1). The formation of the three-phosphate bridge in the VSV mRNA's, as $\text{G}^{\alpha\beta}/\text{p}^{\alpha}\text{A}$, indicated that the mRNA's may have been synthesized by a processing mechanism in which capping involved $\text{G}^{\alpha\beta\gamma}/\text{pp}^{\alpha}\text{A}$ and 5'- αA ends from newly processed mRNA. Alternatively, the mRNA species may indeed be initiated RNA molecules with pppA ends, and capping with GTP may proceed by removal of the β, γ -phosphates of adenosine and the γ -phosphate of guanosine. This reaction, if operative, is clearly different from the reactions in the reovirus, vaccinia virus, and CPV systems described above, which have such capped structures as $\text{G}^{\alpha}/\text{p}^{\beta\alpha}\text{N}$. Recently, 5'-ppA-ended VSV mRNA fragments have been detected in vitro (D. Testa, P. K. Chanda, and A. K. Banerjee, unpublished data), suggesting that in the VSV system removal of the β, γ -phosphates of adenosine and the γ -phosphate of guanosine may occur at least in vitro. It is interesting to note that unlike reovirus and vaccinia virus, the capping of VSV mRNA in vitro is not inhibited by PP_i and P_i (17). Moreover, the mRNA synthesized in the presence of GMPPNHP (replac-

ing GTP) contains the normal cap, $\text{G}^{\alpha\beta}/\text{p}^{\alpha}\text{A}$

(18). It was later confirmed that purified VSV contains a phosphohydrolase that can convert GTP to guanosine diphosphate (GDP) and that the latter can be converted to GTP by a virion-associated nucleoside diphosphate kinase (251). Recently, by using a fish rhabdovirus it was shown that, unlike VSV, in the presence of GMPPNHP the cap structure was $\text{G}^{\gamma}/\text{p}^{\beta\alpha}\text{A}$, indicating that an alternate capping reaction may be operative in this system (96). Like CPV, AMPPNHP is not used in place of ATP in the in vitro reaction for RNA synthesis. This is probably because initiation of VSV mRNA involves the synthesis of a leader RNA which is not capped and contains the 5'ppACG... sequence. This step may require ATP containing hydrolyzable β, γ -phosphates (251).

Influenza virus. The mechanism by which a 5'-terminal cap is added to influenza virus mRNA appears to be unique. It was observed that the mRNA's synthesized in vitro by the virion-associated RNA polymerase in the presence of AdoMet with or without primers, such as ApG (193), did not contain cap structures. In contrast, the viral mRNA's isolated from infected cells were capped and methylated and contained the sequences $^7\text{mGpppA}^{\text{m}}\text{p}$..., $^7\text{mGpppN}^{\text{m}}\text{A}^{\text{m}}\text{p}$..., and $^7\text{mGpppG}^{\text{m}}\text{p}$... (134). The predominant species of caps contained methylated adenosine. These results suggested that complementary RNA synthesis in infected cells initiates preferentially with adenosine and that the RNA polymerase in purified virions, unlike the RNA polymerases in other RNA viruses, did not associate with the enzymes for cap structure formation. This apparent paradox was resolved when it was found totally unexpectedly that the virion-associated RNA polymerase reaction is stimulated by exogenously added globin, reovirus, and other capped mRNA's (31, 32) and that a portion of the 5'-terminal region of the mRNA (approximately 10 to 15 nucleotides) is donated to the influenza virus mRNA in vitro. Thus, it appeared that the purified virion of influenza virus contains enzymes that can incorporate a specific 5'-terminal portion of capped and methylated mRNA's into the nascent transcripts (192) either by splicing onto the influenza virus-specific mRNA synthesized in vitro or by cleavage and extension, the added mRNA acting as a primer for RNA synthesis. This unique enzyme system can recognize only capped mRNA's containing ^7mG since chemically de-capped globin mRNA (32) and blocked but unmethylated reovirus mRNA's do not act as

primers for in vitro mRNA synthesis. Recently, it has been shown that a similar mechanism probably also operates in vivo (133). Influenza virus-specific complementary RNA from infected cells contains a nonviral stretch of 10 to 15 nucleotides cannabilized from cellular mRNA's. These results suggest that host cell mRNA's serve as primers for viral RNA transcription in infected cells, accounting for the previously observed requirement of functional host nuclear RNA polymerase II for influenza virus replication (133).

It is clear from the studies on the viral systems described above that the presence of a virion-associated RNA polymerase greatly facilitated elucidation of the capping reaction. In those viruses that do not contain a virion polymerase, such as adenovirus, simian virus 40, polyoma virus, herpesvirus, etc., the mechanism of the capping reaction has been studied by nucleotide sequence determination of complementary RNAs pulse-labeled in vivo. By direct sequence determinations of the late promoter regions and the 5'-terminal portions of the nuclear and cytoplasmic transcription products formed during late times after adenovirus infection, Ziff and Evans (278) precisely mapped the transcripts on the promoter region. It was concluded that the capped termini of nuclear and cytoplasmic RNAs and the late promoter map together, suggesting that the initiating residues of the primary transcript are precursors of the capped terminus. From similar studies with simian virus 40 (37) and polyoma virus (72) late mRNA's, the viral transcripts also appear to be initiated molecules that are capped subsequently. In contrast to the adenovirus system, the mRNA's of these two viruses contain highly heterogeneous 5'-terminal sequences (37, 72, 89, 97). These results indicate that the RNA polymerase may initiate transcription over a range of nucleotides on the promoter region. The precise reasons for this heterogeneity in the 5'-terminal cap sequence are not known. Recently, similar heterogeneity in cap sequences has been shown in the early mRNA's of adenovirus-infected cells (99). Interestingly, one of the six early promoter sequences contains $^7\text{mGpppU}^{\text{m}}\text{pN}_2$ as the cap structure, indicating that a pyrimidine can be present in the N_1 position in viral mRNA's (100).

Cellular mRNA's

As described above, cellular mRNA's contain the cap structure $^7\text{mGpppN}_1^{\text{m}}$, where N_1 is G^{m} , $\text{N}^{\text{m}}\text{A}^{\text{m}}$, A^{m} , methylcytidine, or methyluridine. It has been generally assumed that purines in the N_1 position indicate that caps are put on initiated RNA chains and that when the N_1 base

is a pyrimidine, caps are formed on cleaved molecules (218). This assumption is mainly derived from the observation that in procaryotic systems, the RNA polymerase initiates chains predominantly with purines (153). In fact, all bases are used for initiation, but purines are favored 5 to 10 to 1 over pyrimidines, depending on the DNA template (153). Thus, RNA polymerase II of eucaryotic cells would also presumably initiate mainly or exclusively with purines. This assumption may no longer be tenable since initiation with pyrimidines has now been shown to occur in at least in one bacteriophage operon (209) and at a second promoter for ribosomal RNA synthesis in *Escherichia coli* (275). Nevertheless, it has not been demonstrated conclusively in eucaryotes whether RNA chains may indeed initiate with pyrimidines. A detailed analysis of the 5'-terminal structure of the hnRNA from mouse L-cells (the presumed precursor of mRNA) revealed the presence of purine tri- and diphosphates, such as pppG, pppA, ppG, and ppA (219), as well as the four varieties of cap structures described above. These results indicate that the 5' portions of some of the primary transcripts (those starting with purines) are directly converted to caps. Similar uncapped 5' termini have also been found in pre-mRNA molecules of Ehrlich carcinoma cells (15). The RNA chains containing pyrimidines may be generated by either (i) an initiation process or (ii) cleavage at specific sites on hnRNA. In the former case, capping and initiation may be concurrent, and in the latter capping may occur on RNAs containing monophosphates at the 5' termini. In an earlier report, Schibler and Perry (218) found large amounts of pUp in addition to ppUp and ppCp at the 5' termini of the hnRNA. It was proposed that the mRNA segments derived by cleavage could be converted by a cellular kinase action to diphosphate-terminated pyrimidines, which would then condense with GTP to form a pyrimidine-containing cap structure. Later it was found that no pyrimidine diphosphates were present in the hnRNA, thus negating this model (219). Although the biosynthesis of the pyrimidine-containing cap structure remains unclear, the existence of enzyme activities in mammalian cells which form caps by condensation of GTP with diphosphate-terminated RNA lends credence to the idea that probably all capped RNAs (including those with pyrimidine-containing caps) are initiated, primary transcripts (see below).

Recent experiments (214) have shown that in cells labeled by brief exposure to [*methyl- ^3H*]-AdoMet the majority of the labeled 5'-terminal cap 1 structures were in hnRNA molecules hav-

ing a chain length of about 750 nucleotides or less. After longer labeling times, the proportion of cap 1 structures in hnRNA molecules longer than mRNA molecules was increased to more than 50% of the total. The cap structures in both long and short hnRNA chains contained all four 2'-O-methylated nucleotides in the N₁ position in about the same proportion as in mRNA. These results may indicate that hnRNA chains can indeed initiate with pyrimidines and that capping occurs very close to or at the start of chain formation.

The mechanism of capping in cellular and viral systems has also been approached by using isolated nuclei to study transcription and capping in vitro. Groner and Hurwitz (94) reported that the RNA synthesized in vitro in the presence of AdoMet by HeLa cell nuclei contained a variety of capped and methylated 5'-terminal structures, such as ⁷mGppN^mp, ⁷mGppN₁^mpN₂^mp..., ⁷mGpppN₂^mp..., and ⁷mGpppN₁^mpN₂^mp. The relative amounts of N₁^m were as follows: guanosine, 45%; adenosine, 25%; uridine, 20%; and cytosine, 10%. Thus, in the in vitro system, in addition to the cap 1 structures, diphosphate-containing capped structures and cap 2 structures were also found. In contrast, in the hnRNA isolated from the nucleus, only cap 1 structures were found, and cap 2 structures were obtained only in the mRNA's isolated from the cytoplasm (73, 187, 215). Clearly, in contrast to intracellular nuclei, in isolated nuclei methyltransferases involved in methylating N₁ and N₂ bases are present and active. Using a subcellular system isolated from mouse L-cell nuclei, Winicov and Perry (270, 271) have also shown that cap 1 and cap 2 structures were present in the in vitro transcripts. It was further shown that cap formation in large nuclear RNA species synthesized in vitro was closely associated with transcription, as indicated by α-amanitin sensitivity and a requirement for the presence of all four ribonucleoside triphosphates. Recently, Groner et al. (92) showed that [β-³²P]ATP can be incorporated into the 5'-terminal capped structure of RNA synthesized in vitro by HeLa cell nuclear homogenates. Approximately 10% of the RNA chains were initiated in vitro with [β-³²P]ATP, and this value decreased considerably when α-amanitin was present in the reaction mixture. These results indicate that, at least for those RNA chains containing adenosine in the N₁ position, initiation and capping occur by condensation of pG from GTP on either a diphosphate or triphosphate 5' end. It was further observed that in contrast to triphosphate caps, in which the N₁ base is any one of the four 2'-O-methylated derivatives, the diphosphate caps

(⁷mGppN^mp) contain exclusively G^m at the N₁ position. It is not clear whether these cap-containing RNA species are artifacts of the in vitro reaction or whether capping enzymes present in the nuclei may carry out reactions which have not been observed in vivo.

Using isolated nuclei from adenovirus-infected cells, Manly et al. (155) demonstrated that adenovirus-specific RNA was synthesized in vitro and that the RNA species originated at a map position expected for late mRNA in vivo promoters (i.e., map position 16.5). Moreover, it was observed that the 5' termini of the RNAs were capped with ⁷mG and that the amount of radioactivity in 7-methylguanosine monophosphate was equivalent to the amount of radioactivity in any other single nucleotide in the 5'-terminal RNase T₁ fragment. Since a large amount of the transcription observed arises from elongation of nascent chains initiated in vivo, the possibility existed that [α-³²P]GTP was incorporated into the 5' termini of the RNA chains already initiated in vivo. Under these conditions, a greater than equimolar amount of radioactivity in 7-methylguanosine monophosphate would have been obtained; thus, the results indicate that post-transcriptional capping does not occur at detectable levels and that the capping and transcription initiation processes are tightly linked.

ENZYMES INVOLVED IN THE CAPPING REACTIONS

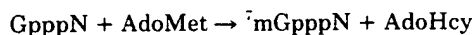
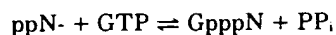
From the structure of the 5' cap and the mechanism of its formation, it became apparent that multienzyme systems are involved in the formation of fully methylated capped 5' ends. The enzymes involved in the cap formation are as follows. (i) mRNA guanylyltransferase, involved in the first step of the capping reaction, catalyzes the formation of the cap structure by using GTP that is linked through a triphosphate bridge to the 5' end of the RNA. To carry out this reaction, two other enzymatic activities appear to be involved; these are (a) an enzyme that converts 5'-phosphate-terminated RNA to 5'-di- and triphosphate-terminated RNAs, which in turn can be used for capping with GTP (239) and (b) an RNA triphosphatase activity that converts triphosphate-terminated RNA to diphosphate-terminated RNA (the latter is then used as a substrate for capping) (257). (ii) mRNA (guanine-7-)methyltransferase catalyzes the transfer of a methyl group from AdoMet to the position 7 of the capping guanosine residue. (iii) 2'-O-Methyltransferase activity methylates the 2'-O-ribose moieties of the N₁ nucleotides of the mRNA's. (iv) A methyltransferase activity spe-

cifically catalyzes the transfer of a methyl group from AdoMet to the N⁶ position of a 2'-*O*-methyladenosine residue at the N₁ position of the capped 5' end of mRNA.

All of the above-described enzymes have been purified from vaccinia virions by Moss and his co-workers (19, 20, 65, 157-159) with the exception of the last enzyme, which was purified by them from HeLa cells (120). RNA (guanine-7-)methyltransferase was also purified from HeLa cells (66). Purifications of some of these enzymes have also been carried out in the laboratory of Hurwitz (164, 165). Recently, both guanylyltransferase and methyltransferase activities were purified from rat liver nuclei (163). The properties and specificities of these enzymes are described below.

mRNA Guanylyltransferase and mRNA (Guanine-7-)Methyltransferase

Martin et al. (159) first reported the isolation and purification of these enzymes from vaccinia virions. They took advantage of the selective binding of these activities to homopolyribonucleotides to achieve a 200-fold increase in specific activity. Both activities remain together during affinity or ion-exchange chromatography. The purified enzyme has a molecular weight of 127,000 and contains two polypeptides of molecular weights 95,000 and 31,400 in a molar ratio close to 1:1, indicating that both activities are components of an enzyme system. This system specifically catalyzes modification of the diphosphate-containing 5' termini of mRNA, poly(A), or polyguanydic acid (157, 158):



The enzyme prefers Mg²⁺ over Mn²⁺, and the optimum pH is 7.8. Studies on the donor and substrate specificities of this enzyme complex revealed that diphosphate-terminated polyribonucleotides (such as pp[N]_n, where N can be any base) are preferred and that monophosphate termini are not used. Although mononucleotides (ppN) are capped, trinucleotides (ppNpNpN) are better substrates, and slightly longer polyribonucleotides might be optimal (157). GTP, inosine triphosphate, and deoxyguanosine triphosphate, but not ⁷mGTP, can be used as donors.

Monroy et al. (164) reported an approximately 10,000-fold purification of the same activities from purified vaccinia virus. Slightly different properties were observed. The molecular weight of the native protein is 120,000; a major band of molecular weight 95,000 is released in sodium dodecyl sulfate-polyacrylamide gel electropho-

resis, along with minor bands having molecular weights of 59,000 and 28,000. However, when the native guanylyltransferase is purified by non-denaturing polyacrylamide gel electrophoresis, recovered, iodinated, and electrophoresed on a polyacrylamide gel containing sodium dodecyl sulfate, a major polypeptide band of molecular weight 59,000 is observed. This enzyme specifically uses 5'-triphosphate-ended RNA chains [ppp(A)_n] (165). The apparent *K_m* for termini of ppp(A)_n is 0.2 μM, indicating a high affinity of the enzyme for the substrate. During guanylation of ppp(A)_n, P_i and PP_i are both released in a 1:1 stoichiometry with cap formation. The reason for the apparent discrepancy between these results and those described by Moss and his colleagues (157, 158) are presently unclear. However, Venkatesan and Moss (257) have recently detected an additional enzyme activity (RNA triphosphatase) in purified vaccinia virions. This activity also appears to be a component of the guanylyltransferase enzyme complex. In addition, this enzyme activity is involved in the removal of γ-phosphate from triphosphate-ended RNA chains, converting the 5' end to a diphosphate, which in turn is used by the enzyme as the principal substrate. A similar mechanism has been proposed previously for the capping of reovirus mRNA's in vitro (83).

Spencer et al. (239) have reported isolation of a 5'-phosphate-phosphate polyribonucleotide kinase activity from vaccinia virus cores; this activity catalyzes the conversion of 5'-phosphate and 5'-diphosphate termini of RNAs to 5'-triphosphate forms. 5'-Hydroxyl RNA or DNA and 5'-phosphorylated DNA are not phosphorylated by this enzyme. In a system that coupled 5'-phosphate polyribonucleotide kinase and guanylyltransferase, p(A)_n and pp(A)_n were capped. The guanylyltransferase by itself, however, can only cap ppp(A)_n. The precise role of the 5'-polyribonucleotide kinase activity in the capping process is not known, but this enzyme may be involved in the capping of mRNA's that are formed by cleavage of pre-mRNA (219) to form 5'-phosphate termini, which are then converted to capped molecules.

2'-*O*-Methyltransferase

This activity has been purified from vaccinia virions approximately 350-fold. It catalyzes the transfer of a methyl group from AdoMet to the 2'-OH of the penultimate nucleoside of the capped mRNA, such as conversion of ⁷mGpppG in bromo mosaic virus RNA to ⁷mGpppG^m (19). The molecular weight of the enzyme is 38,000. RNAs ending on pN, ppN, or GpppN are ineffective substrates for this enzyme (20). poly(A)

and polyinosinic acid ending in $\bar{m}GpppN$ are excellent substrates, whereas capped polyguanosinic acid, polyuridylic acid, and polycytidylic acid are poor substrates for the enzyme. The low ability of the enzyme to use dinucleotides as substrates (for example, $\bar{m}GpppG$) suggests that a longer polyribonucleotide chain is required. This activity is extremely sensitive to AdoHcy. With only $1 \mu M$ AdoHcy in the presence of $2.5 \mu M$ AdoMet, the activity is virtually abolished. This is in contrast to the VSV system, in which 2'-O-methyltransferase activity is more resistant to AdoHcy than (guanine-7-)methyltransferase (250).

Guanylyltransferases and Methyltransferases from Eucaryotic Cells

Like the enzymes in vaccinia virus, the enzymes involved in the capping reaction in eucaryotic cells have also been detected and partially purified. A soluble extract prepared from HeLa cell nuclei has been shown to catalyze the 5'-terminal modification of RNA and synthetic polyribonucleotides to form $\bar{m}GpppA$ and $\bar{m}GpppG$ structures (66, 265). The substrate RNA molecule appears to require the presence of at least two terminal phosphates. A polyribonucleotide with only a single 5'-terminal phosphate is not capped unless a second phosphate is added. Thus, it appears that the first step of the capping reaction in eucaryotic cells may be similar to that in the vaccinia virus and reovirus systems. More recently, Venkatesan et al. (S. Venkatesan, A. Gershowitz, and B. Moss, personal communication) have purified this enzyme 1,000-fold from HeLa cell nuclei. It has a molecular weight of 48,500 and shows optimal activity at pH 7.5 in the presence of Mn^{2+} or Mg^{2+} . Using this purified enzyme, Venkatesan and Moss have shown that RNA with ppC at the 5' end (a transcription product of phage λ c17 DNA [209]) is also capped by purified mRNA guanylyltransferase, indicating that pyrimidine-ended as well as purine-ended RNAs can serve as acceptors. This result is consistent with the presence of a pyrimidine in the N_1 base of the cap in eucaryotic mRNA's and also suggests that pyrimidine-containing caps may also be part of initiated RNA molecules. Recently, an mRNA guanylyltransferase purified from wheat germ has also been shown to use only diphosphate-ended RNA (J. Keith, A. Gershowitz, S. Venkatesan, and B. Moss, personal communication).

Unlike vaccinia virus, the HeLa cell RNA (guanine-7-)methyltransferase is clearly separable from the guanylyltransferase activity (66). This enzyme specifically methylates the 5'-terminal guanosine of RNA chains ending in

GpppG or GpppA. It differs from the vaccinia virus enzyme in the following respects. (i) The HeLa cell enzyme exhibits exclusively methyltransferase activity, whereas the vaccinia virus is part of an enzyme complex that also includes guanylyltransferase activity (see above). (ii) GTP and GDP are not substrates for HeLa cell enzymes, whereas these compounds can be methylated to a small extent by the viral enzyme. (iii) The HeLa cell enzyme has a molecular weight of 56,000, whereas the molecular weight of the viral enzyme is 127,000.

As discussed above, in viral and cellular mRNA's synthesized in vivo (in contrast to in vitro mRNA in the case of viruses) adenosine residues in cap structures exist largely as the unusual dimethylated nucleoside N^6 mA^m (Table 1). Consistent with this observation, an enzyme activity (molecular weight, 65,000) was observed in the postribosomal supernatant of HeLa cells; this activity specifically methylates the N^6 position of 2'-O-methylated adenosine in the cap structure (120). Less activity was found with RNA ending in $\bar{m}GpppA$, and barely detectable activity was found with RNAs ending in GpppA and ppA- or oligonucleotides of the type $\bar{m}GpppA^m$ pN or $\bar{m}GpppApN$. The latter results indicate that some extension of RNA is required before the methylation of the N-6 position of adenosine may occur. The failure of this enzyme to methylate internal RNA segments makes it likely that a separate methyltransferase, not isolated so far, catalyzes the formation of internal m^aA in eucaryotic mRNA's (Table 1).

Recently, separate guanylyltransferase and RNA (guanine-7-)methyltransferase activities have been purified from rat liver nuclei (163). They have molecular weights of approximately 65,000 and 130,000, respectively. For the guanylylation reaction, dithiothreitol is essential, and Mn^{2+} (2 mM) is twice as effective as Mg^{2+} (8 mM). The capping reaction appears to involve condensation of guanosine monophosphate to a diphosphate-terminated ribopolymer [such as pp(A)n, ppGpCpC, etc.]. The triphosphate-terminated ribopolymers are capped at much lower rates than their diphosphate-terminated counterparts. In this respect, the mode of action of this enzyme is similar to the vaccinia virus enzyme reported by Martin and Moss (157, 158). Recently, Bajszar and co-workers (16) have reported that 30S ribonucleoprotein particles from rat liver nuclei contain (guanine-7-)methyltransferase and 2'-O-methyltransferase in addition to guanylyltransferase.

RNA (guanine-7-)methyltransferase has also been partially purified from *Neurospora crassa*

(87). It catalyzes the transfer of a methyl group from AdoMet to an RNA containing a 5'-terminal unmethylated capped structure. *N. crassa* poly(A)⁺ RNA and reovirus mRNA are equally good as substrates. No 2'-O-methyltransferase activity is associated with this enzyme, which is consistent with the absence of 2'-O-methylation in *N. crassa* mRNA (220). Although not purified, a similar methyltransferase activity has been demonstrated in the extracts of wheat germ (173, 184; J. Keith and B. Moss, personal communication), *Artemia salina* (174), calf thymus (141), and embryonic chicken lens (136).

Recently, two separate mRNA (nucleoside-2'-O-)-methyltransferases from HeLa cells have been purified; one of these completes formation of cap 1 structures and the other yields cap 2 structures with suitable acceptors (S. Langberg and B. Moss, personal communication).

Enzymes That Cleave the Cap Structure

Enzymes that cleave the cap structure into ⁷mGp and pN^m-containing mRNA have been isolated and purified free from RNase from tobacco tissue culture cells (228) and potato (276). The nucleotide pyrophosphatase purified from potato has been used to remove the caps from reovirus, rabbit globin, and *A. salina* mRNA's for studies on the effect of caps on mRNA translation (276). The corresponding enzyme from tobacco cells was used similarly to decap CPV mRNA (226, 229). Moreover, after removal of the cap from tobacco mosaic virus RNA, it was shown that the ability of the RNA to reassemble with the coat protein was unchanged, but infectivity was abolished (181). By using tobacco pyrophosphatase, the blocking nucleotide of rabbit globin mRNA was removed, and the mRNA was subsequently sequenced by the polynucleotide kinase method, using γ -ATP (63).

An enzyme activity was also detected in HeLa cell extracts; this activity specifically cleaved the dinucleotide ⁷mGpppG^m to ⁷mGp and ppG^m (180). It did not hydrolyze the corresponding unmethylated structure or ring-opened cap derivative. However, this enzyme failed to cleave the cap structure present in mRNA. It appears to require for activity capped oligonucleotides shorter than 10 bases (179). The low-molecular-weight nuclear hypermethylated capped RNAs were not substrates for the enzyme. The function of this enzyme in eucaryotic cells is not known. One of the proposed functions is as a scavenger by which accumulated cap structures are destroyed during degradation of mRNA's, thus avoiding inhibitory effects of caps on ribosome binding and eventually facilitating translation of capped mRNA (179). A similar nucleotide py-

rophosphatase activity has also been detected in embryonic chicken lens (135). It releases ⁷mGp from the cap structure ⁷mGpppN (where N is adenosine, A^m, G^mpCp, A^mpApC, or A^mpApCpApG) (137). Phage T4-induced polynucleotide kinase has also been reported to cleave the PP_i bond from a cap structure, but this has not been confirmed (4).

BIOLOGICAL ROLE OF THE CAP STRUCTURE

The presence of the unconventional cap structure in eucaryotic mRNA's prompted investigations to determine its role in mRNA function. It was quickly recognized that the cap structure carrying the polar ⁷mG moiety may play a significant role in the interaction of mRNA with ribosomal RNA or its protein components during initiation of protein synthesis. Moreover, the 5'-terminal cap structure of mRNA may be resistant to the action of 5'-exonuclease action, thus imparting stability to the mRNA's within the cell. Finally, the capping process may be directly involved in the biosynthesis of mRNA. These areas covering the possible roles of the cap structure have been investigated in great detail, and the results strongly indicate that cap structures may indeed play an important role in conferring higher translational ability and stability to mRNA's and in addition may also be directly related to the transcription process.

Protein Synthesis

Both et al. (28) were the first to demonstrate that the cap structure in mRNA was required for efficient translation of VSV and reovirus mRNA's in a wheat germ cell-free protein-synthesizing system. The corresponding uncapped (reovirus) and capped and unmethylated (VSV) mRNA's were virtually untranscribed in the same extract. Similar results were obtained when the translational abilities of capped reovirus, VSV, and globin mRNA's were compared with the corresponding mRNA's after chemical removal of the cap structures from their 5' ends (173). Subsequently, Both et al. (29) observed that the cap structure facilitates efficient binding of reovirus mRNA's to 40S ribosomal subunits during initiation of protein synthesis. After these discoveries, a great deal of data have been accumulated over the years; the role of the cap structure in translation has been studied extensively by using a number of different mRNA's from viral and eucaryotic sources and a variety of cell-free protein-synthesizing systems. The recent reviews by Filipowicz (70) and Kozak (130) have dealt comprehensively with the functions of the cap structure in protein synthesis

and its possible involvement in the capacity of ribosomes to select the initiation regions in mRNA's. To avoid repetition, I have summarized below the observations already reviewed and added some new interesting findings regarding the role of mRNA cap structures in eucaryotic protein synthesis.

From a vast body of reports published over the years concerning the function of the cap structure in mRNA translation, some distinct patterns emerged.

Uncapped or unmethylated mRNA's are poor templates for protein synthesis in vitro. This was shown either by using standard in vitro synthesized mRNA's that lacked the cap structure or by removing the cap structure from the mRNA by chemical or enzymatic methods. The translational abilities of these mRNA's and their abilities to bind to initiation complexes were then tested in a variety of cell-free protein-synthesizing systems. The mRNA's tested were from reovirus (104, 146, 173, 175, 276), vaccinia virus (176, 185, 259), VSV (151, 175, 206, 254), CPV (226), tobacco mosaic virus (177, 226, 272), brome mosaic virus (225), globin (104, 149, 173, 226, 272, 276), protamine (86), bovine parathyroid (123), hen ovalbumin (222), and *A. salina* (174). The results indicated that virtually all mRNA's that lacked 7mG were ineffective templates for protein synthesis. The observed translational deficiency of mRNA lacking 7mG was more pronounced in wheat germ extract compared with reticulocyte lysates and ascite extracts. It was further observed that wheat germ extracts and reticulocyte lysates responded differently to changes in salt concentrations when translating uncapped mRNA's (25, 41, 124, 185, 272). It is not clear whether there is an intrinsic difference in the components used for chain initiation or whether the extracts differ only in the concentrations or activities of some common initiation components. Although other conditions, such as mRNA concentration (110, 146, 225, 272), initiation factors (104, 116), and temperature (259), favorably affect the translation of uncapped mRNA's under optimum conditions, the importance of the cap for translation was evident.

Recently, the role of the cap structure in translation of procaryotic mRNA's in eucaryotic cell-free systems has been studied. Various procaryotic mRNA's have been translated with accuracy in both mammalian and wheat germ cell-free systems to produce functional protein products (11), but the efficiency of translation was significantly higher in the procaryotic extract. Using vaccinia virus capping enzyme, Paterson and Rosenberg (184) were able to cap the in

vitro mRNA's coding for λ *cro* gene. The capped mRNA's were translated at relatively high efficiencies in wheat germ extract (compared with capped rabbit globin mRNA), and this efficient translation was absolutely dependent on the modification of the λ transcripts by addition of the cap structure. Like other capped mRNA's, *cro* protein translation was inhibited by the cap analog 7mGpppA. These results suggest that the procaryotic mRNA's contain all of the information necessary for efficient recognition and initiation by eucaryotic translation components, except for the cap structure.

7-Methylguanosine facilitates binding of mRNA's to ribosomes. Although the translation of capped and uncapped mRNA's in vitro depended on various experimental parameters, the ribosome binding studies were unambiguous. In experiments with capped polynucleotides, such as 7GpppG^mC(U)_n and 7mGpppG^mC(A,C)_n, it was shown that these polymers were able to bind more efficiently to 40S ribosomal subunits than the corresponding uncapped ribopolymers (30). Capped mRNA's were also more efficient than their uncapped counterparts in binding to ribosomes to form stable initiation complexes (151, 175, 176, 206, 259). The addition of a cap structure to uncapped mRNA by vaccinia virus enzyme increased the rate and extent of binding of vaccinia virus mRNA to wheat germ ribosomes (176). Moreover, in a mixture of mRNA's containing capped and uncapped mRNA's, capped mRNA was found to be rapidly and selectively associated with ribosomes. (175, 176). The notable exceptions are the mRNA's that normally lack the cap (e.g., poliovirus, satellite tobacco necrosis virus, cow pea mosaic virus) but nevertheless bind to ribosomes and are translated efficiently in in vitro systems. Uncapped satellite tobacco necrosis virus RNA was apparently translated equally efficiently when it was enzymatically capped (232). The latter examples indicate that in these systems cellular factors play a primary role in the translation process (see below).

From experimental data relating binding of the 40S and 80S initiation complexes to the 5' ends of a number of mRNA's, Kozak and Shatkin (130, 132) postulated a "scanning model" in which the 40S ribosomal subunit first attaches to the 5' end of a message, irrespective of sequence, and subsequently advances until it encounters the first AUG triplet, at which point the 40S ribosome stops and the 60S subunit joins. The presence of 7mG probably facilitates the initial binding with 40S ribosome. This translation process in eucaryotic systems is clearly different from the polycistronic procar-

yotic mRNA's, in which the ribosomes can attach to multiple AUG codons in the mRNA. Eucaryotic mRNA's, on the other hand, presumably contain one functional AUG codon to account for their monocistronic nature. This model may account for the inability of the ribosome to attach to an internal AUG codon and is also compatible with the observed variability in the location of the AUG codon within the 5'-proximal regions of different messages with varying neighboring sequences. The nucleotide sequences of the 5'-proximal regions of eucaryotic mRNA's (130) indicate that the initiator codon AUG is located at variable distances, ranging from 10 to 100 bases from the 5'-terminal cap. The interaction of the 5' terminus of the message with the 40S subunit appears to be more important than the position or location of the AUG codon in the mRNA. In support of this model, Kozak (131) has recently demonstrated that eucaryotic ribosomes failed to attach to the AUG codon when present in a circular mRNA. Circularization of pUpG(pA)₄₀₋₈₀ or of poly(A,U,G) by T₄ RNA ligase virtually abolished the ability of the template to bind to wheat germ or reticulocyte ribosomes. In control experiments, linear forms of each polymer were able to form initiation complexes in wheat germ and reticulocyte extracts, and the circular molecules that failed to bind to wheat germ or reticulocyte ribosomes were able to bind to *E. coli* ribosomes. These results are compatible with the proposed mechanism by which eucaryotic ribosomes enter at the open 5' terminus of a message by a threading mechanism, with translation beginning at the first AUG codon encountered by the ribosome. The precise function of the cap structure in this process is not clear since, as described above, uncapped mRNA's also attach to ribosomes and are translated, although at lesser efficiency.

The role of ⁷mG in translation has also been tested by using a polycistronic procaryotic mRNA. Rosenberg and Paterson (209) used a high-molecular-weight polycistronic *in vitro* λ DNA transcript containing the 5'-terminal *cro* gene in the RNA. When a cap structure was added to the 5' end of the RNA before translation in wheat germ extract, efficient translation of only the *cro* gene occurred. The inability to detect protein synthesis from any of the cistrons located internally in the transcript suggests that the eucaryotic ribosome interacts with the cap structure, which subsequently allows recognition of the first AUG located close to the 5' end. Using a similar translation system, these investigators have also shown that in the *gal* operon-derived polycistronic message only the cap-proximal cistron (*galE*) was translated *in vitro*. Future experiments in this area should give inter-

esting insights into the nature of the interactions of the cap structures in procaryotic mRNA with eucaryotic ribosomes that allow the 5'-proximal AUG to be recognized in a polycistronic message.

Chemical analogs of cap structures, such as p⁷mG, ⁷mGDP, and ⁷mGpppN^m, inhibit translation and ribosome binding of capped mRNA. Under appropriate ionic conditions these cap analogs inhibited translation of a variety of capped mRNA's in various cell-free extracts (13, 38, 71, 93, 110, 203, 221, 245, 258, 268). These studies indicate that some special structural features of the cap play a significant role in the recognition process during the formation of the initiation complex of mRNA and ribosomes. In a survey of the inhibitory effects of different methylated nucleosides and nucleotides in translation and ribosome binding, some interesting structural requirements for cap recognition were observed. ⁷mG, GMP, 7-methylguanosine 2',3'-cyclic phosphate, and GpppN were not inhibitory to translation, whereas p⁷mG, ⁷mGDP, ⁷mGTP, and ⁷mGpppN^m were potent inhibitors. These results indicate that some interaction between the polar group of ⁷mG and the negatively charged phosphate group(s) of these inhibitors is involved in altering the binding of capped mRNA to ribosomes (71, 111). Replacement of the methyl group by ethyl (7-ethyl)GDP or benzyl (7-benzyl)GDP did not decrease the inhibitory capacity of ⁷mGDP (6), indicating that the maintenance of the positive charge (but not necessarily a methyl group at the N7 position) is essential for the inhibitory effect. This is also borne out by the observation that 7-ethylguanosine-containing capped mRNA's (mRNA's synthesized *in vitro* by reovirus-associated enzymes in the presence of *s*-adenosyl-*l*-ethionine) were essentially equally active in translation and binding to ribosomes compared with their methylated counterparts (80).

Cap-binding protein. Since ribosomal subunits from wheat germ or reticulocyte extracts bound efficiently to the 5'-terminal region of many mRNA's and protected cap-containing sequences against RNase digestion (132), it seemed possible that initiation complexes included proteins capable of binding mRNA (cap-binding protein [CBP]), thus protecting the cap from nuclease action. Activity of this type was detected in an *A. salina* ribosomal high-salt wash by assaying the binding of methyl-³H-labeled ⁷mGpppG^mC to membrane filters (71). By the same technique it was found that preparations of the purified rabbit reticulocyte initiation factors (for nomenclature, see Anderson et al. [12]) eIF-2, -4B, and -5 bound to mRNA (21, 22,

221). The binding of eIF-4B to capped mRNA, but not to encephalomyocarditis virus RNA, which is uncapped, was inhibited by the cap analog p⁷mG. On this basis, it was suggested that eIF-4B recognizes and binds to the cap structure (221). By using the same experimental approach, it was shown that eIF-2 also recognizes the cap structure in mRNA (116). These researchers used the filter binding technique, which may not permit unequivocal identification of polypeptides with cap-binding activity, especially if the cap-binding component is a contaminant with a strong affinity for caps. To detect protein with cap-binding activity, Sonenberg and Shatkin (237) used a cross-linking method by which proteins in the proximity of the 5' end of mRNA in initiation complexes can be detected. Using this method and polyacrylamide gel electrophoresis, Sonenberg et al. (234) detected a 24,000-dalton polypeptide in initiation factors prepared by high-salt washes of rabbit reticulocyte, mouse ascites, and human HeLa cell ribosomes. The CBP can be specifically cross-linked to methyl-³H-labeled capped viral mRNA as defined by ⁷mGDP inhibition. The 24,000-dalton CBP was detected only in purified eIF-3 and -4B among the many factors tested. The strong cap interaction of the CBP was not limited to reovirus mRNA but was also observed with reticulocyte eIF-3 cross-linked to VSV, Newcastle disease virus, and vaccinia virus capped mRNA's (235). The failure to obtain nonspecific binding and cross-linking to the CBP in the presence of ⁷mGDP suggests that this polypeptide has a single site available for cross-linking and that this site recognizes the ⁷mG nucleotide. Recently, the CBP from reticulocyte ribosomes has been purified to homogeneity by chromatographic passage through an affinity resin prepared by coupling the levulinic acid *O*^{2',3'}-acetal of ⁷mGDP to AH-Sepharose 4B (236). The translation of capped mRNA's from Sindbis virus, reovirus, and rabbit globin was stimulated by purified CBP in HeLa cell extracts. The translation of uncapped mRNA's from encephalomyocarditis virus and satellite tobacco necrosis virus was not stimulated under the same conditions (236), indicating that the CBP may interact with the ⁷mG in mRNA before the formation of protein synthesis initiation complexes.

Based on these results, Sonenberg et al. (235) proposed a model for the role of CBP in the promotion of translation of capped mRNA's. The first step in protein synthesis involves the formation of a pre-initiation complex between the 5' end of capped mRNA and the CBP. Since the CBP has a high affinity for eIF-3 (234), the next step is the interaction of the pre-initiation complex containing CBP with the 40S ribosomal

subunits containing eIF-3. After initiation complex formation, the CBP is released for recycling, and its interaction with the 5' end of mRNA is replaced by eIF-3 subunits. This model predicts that eIF-3 or some of its subunits would be present in the 80S initiation complexes, either on the ribosome or on the 5' terminus of the mRNA. Further characterization of the proteins that can be cross-linked to the cap structures of the mRNA's in initiation complexes should clarify the roles of CBP and eIF-3 subunits in capped mRNA translation.

Recently, CBP has been shown to have restoring activity for capped mRNA translation in cell extracts from poliovirus-infected cells. Infection with poliovirus leads to inactivation of the cap-dependent recognition mechanism of mRNA's during protein synthesis initiation. It was observed that the translation of capped mRNA's (e.g., in VSV) was markedly decreased in extracts of poliovirus-infected HeLa cells, whereas poliovirus RNA, which is not capped, continued to function well in vitro (208). The addition of only purified eIF-4B and not other initiation factors to infected cell extracts restored capped mRNA translation without increasing poliovirus synthesis. These results were interpreted as selective inactivation of eIF-4B by poliovirus during infection and thus blocking of host cell protein synthesis (i.e., the translation of capped mRNA's). In view of the stimulatory effect of CBP in translation of capped mRNA's compared with uncapped mRNA's, it was confirmed that the observed restoring activity of eIF-4B was indeed due to the presence of contaminating CBP in the initiation factor (255). The restoring activity was purified from a high-salt wash of rabbit reticulocyte ribosomes, which corresponded to the CBP with regard to electrophoretic mobility, tryptic peptide pattern, and ability to cross-link to the 5'-terminal cap in mRNA. Thus, it appears that at least in the poliovirus system cellular protein synthesis may be inhibited by inactivation of some crucial property of the CBP.

Helentjaris and Ehrenfeld (105) have also shown that a salt wash from uninfected cells stimulated translation of both cell (endogenous translation system derived from HeLa cells) and poliovirus mRNA's, whereas a salt wash from infected cells stimulated the translation only of viral mRNA. This mRNA-discriminating activity was found to be tightly associated with eIF-3 (106). Since CBP has a high affinity for eIF-3 (234), it is possible that the mRNA discriminating activity may be similar, if not identical, to the CBP.

Interesting results were obtained when similar experiments were carried out in reovirus-in-

fect cell extracts. Skup and Millward (231) have shown that extracts from uninfected L-cells translated capped reovirus mRNA at high efficiencies and that the translation was sensitive to $^7\text{mGTP}$. The same extracts translated uncapped reovirus mRNA at low efficiencies. In contrast, extracts from infected L-cells translated uncapped reovirus mRNA at higher efficiencies, and the translation was not sensitive to $^7\text{mGTP}$. These results suggest that like the poliovirus system, reovirus infection renders a component(s) of the protein synthesis machinery inactive, such that translation of capped mRNA's is blocked. However, reovirus mRNA, in contrast to poliovirus mRNA, is capped, and its translation continues unabated during infection. It has been claimed that reovirus mRNA's late after infection are not capped but contain pGp at the 5' end (277). Enzyme activity in L-cell S10 extract rapidly converted diphosphate-ended mRNA's to monophosphate-ended mRNA's. Subsequently, it was shown (230) that the capping and methyltransferase activities become masked in subviral particles isolated from cells at late times after reovirus infection. Thus, the mechanism of host cell shutoff by reovirus infection may be similar to that in the poliovirus system, except that additional controlling features may exist in the former that convert capped viral mRNA to its uncapped form during infection.

mRNA Stability

One of the features that distinguishes eucaryotic mRNA's from procaryotic mRNA's is their longer half-lives. The short-lived procaryotic mRNA's are degraded exonucleolytically from the 5' end during translation (7). Modification at the 5' end of the eucaryotic mRNA's possibly imparts stability by virtue of conferring resistance to the action of such nucleases. One of the reasons for the observed inefficient translation of uncapped mRNA's in *in vitro* cell-free extracts may thus be due to preferential degradation of uncapped compared with capped mRNA's. Several experiments indicated that the cap structure stabilizes mRNA both *in vivo* and in cell-free extracts. Furuichi et al. (77) injected capped reovirus mRNA's into *Xenopus laevis* oocytes and observed that capped mRNA's were more stable than their uncapped counterparts. It was also found that uncapped reovirus mRNA's were preferentially degraded in cell-free extracts isolated from wheat germ or mouse L-cells. Rabbit reticulocyte lysate contained little or no nuclease activity, which is consistent with its loss of transcriptional machinery during differentiation and

possibly accounts for the increased efficiency of translation of uncapped mRNA's in this system. Similar findings confirming the importance of the cap structure in stabilizing mRNA during eucaryotic protein synthesis have been obtained with the mRNA's of tobacco mosaic virus, globin, protamine, and *A. salina* (see above). In each case, the capped mRNA species remained intact, but when they were decapped, they lost their translation capabilities (>90%) in wheat germ cell-free extracts and were degraded exonucleolytically during translation. When capped and uncapped globin mRNA's were injected into frog oocytes, more than 90% of the translatability of uncapped globin mRNA was lost over a 96-h period (149). The capped globin mRNA, on the other hand, was apparently stable since it continued to be translated. These results clearly indicate that the cap structure protects mRNA from degradation and thus increases its stability. There is one unconfirmed report (5) in which removal of ^7mG from globin mRNA by T4-polynucleotide kinase did not alter the translational ability of the mRNA in wheat germ extract.

In contrast to the above-described results, uncapped RNAs, such as those in poliovirus (208) and satellite tobacco necrosis virus (145), which function as messengers in infected cells, are stable and are translated in various model systems. One interpretation of this finding is that at least in the case of poliovirus, the protein linked to the 5' end of the RNA may protect it from nuclease action. However, this argument is weakened by the finding that polysome-associated poliovirus mRNA does not contain the 5'-terminal protein. It is removed from the genome RNA by endogenous proteases present in cell-free extracts. In the case of satellite tobacco necrosis virus, the stabilizing role may be played by a secondary structure at the 5' end of the RNA (232). Although the precise reasons for the stability of these uncapped mRNA's are not known, some special interaction of proteins with the 5' termini may account for their resistance to degradation in infected cells.

mRNA Biosynthesis

Cap structures have been detected in early stages of hnRNA synthesis in nuclei (48). Conversion of precursor transcripts to cytoplasmic mRNA's apparently involves conservation of the cap structure during several processing steps, including 3'-terminal addition of poly(A) and splicing of intervening nucleotide sequences (48). It is still not clear whether cap formation is involved or required for the initiation of mRNA

synthesis. An answer to this question has not been obtained because of the difficulties in controlling nuclear RNA synthesis in order to isolate the primary RNA products in high yields. As discussed above, the mechanisms of cap formation have been studied in detail in viral systems, and in some cases capping and mRNA synthesis are tightly coupled. For example, VSV mRNA's synthesized *in vitro* are always capped, and efforts to find conditions for uncapped mRNA formation have failed (17). Thus, it appears that in the VSV system capping may be essential for the synthesis and eventual chain completion of mRNA's. Although not obligatory, influenza virus may use capped primers for mRNA synthesis *in vitro* (32), but *in vivo* (133) capping appears somehow directly related to the biosynthesis of influenza virus mRNA and thus to viral replication.

CPV is a unique system, in which the capping process appears to be directly involved in mRNA transcription. The addition of AdoMet stimulates *in vitro* mRNA synthesis by the virion-associated RNA polymerase. More interestingly, AdoHcy, a competitive inhibitor of AdoMet, also stimulates RNA synthesis to the same extent as AdoMet (76, 160). Methylation of the cap apparently is not required for mRNA synthesis, but some structural features of AdoMet appear to be involved in the transcription complex and cause activation of RNA polymerase at the initiation site of the genome. This hypothesis is supported by the finding that the apparent K_m for ATP for the initiation of CPV mRNA synthesis is greatly reduced by increasing concentrations of AdoMet. It has been proposed that AdoMet may interact with a methylase subunit in the CPV transcription complex, resulting in an allosteric conformational change that lowers the apparent K_m for initiating ATP and activation of the RNA polymerase (85). Recently, Wertheimer et al. (267) have studied the effects of various AdoMet analogs on CPV transcription *in vitro* in an effort to understand which structural features of AdoMet are important for stimulation of RNA synthesis. It appears that those analogs that generally inhibit methyltransferase also stimulate RNA synthesis. Furthermore, analogs which do not inhibit methyltransferase fail to stimulate mRNA synthesis. These findings also indicate that some interaction between AdoMet and the methyltransferase turns on CPV mRNA synthesis. Stimulatory effects of AdoMet on *in vitro* transcription of a fish rhabdovirus have also been reported (212). More detailed studies in these viral systems should elucidate further the role of capping in mRNA synthesis.

Inhibitors of methyltransferases have been used to understand the role of methylation in mRNA synthesis, transport, and translation *in vivo*. Kaehler et al. (115) have shown that *in vivo* *S*-tubercidinyl homocysteine effectively inhibits ribose 2'-*O*-methylation of the N_1 nucleoside of the cap and base N^6 adenosine methylation in internal positions of Novikoff hepatoma mRNA's. Methylation of 5' blocking guanosine was not affected, and the cytoplasmic mRNA's contained predominantly cap 0 structures. Since this inhibitor did not affect the viability of the cell, it was concluded that ribose methylation or internal adenosine methylation was not needed for mRNA transport from the nucleus or subsequent translation in cytoplasm. Similarly, Dimock and Stoltzfus showed that cycloleucine, a competitive inhibitor of ATP:L-methionine *S*-adenosyltransferase, blocks ribose methylation and internal methylation (but not 7-methylation of guanosine) of avian sarcoma virus genome RNA in chicken embryo cells (56). Viral replication was virtually unaffected under these conditions, indicating that ribose and internal methylation are not required for avian sarcoma virus RNA synthesis, processing, and transport. Similar results were obtained by Bachellerie et al. (14) in Chinese hamster ovary cells treated with cycloleucine. In contrast, Robert-Gero et al. (201) reported that 5'-deoxy-5'-isobutyryl-adenosine, an analog of AdoHcy, inhibited by more than 90% the production of the Schmidt-Ruppin strain of Rous sarcoma virus. These results suggest that 5'-deoxy-5'-isobutyryl-adenosine may inhibit the 7-methylguanosine methyltransferase which may be essential for mRNA synthesis. In fact, Jacquemont and Huppert (114) have shown that 5'-deoxy-5'-isobutyryl-adenosine inhibited mG methylation of viral mRNA, whereas m⁶A methylation was not affected in herpes simplex virus type 1-infected cells. Recently, the antibiotic Sinefungin has been shown to inhibit Newcastle disease virus replication and also the 7-methylguanine and 2'-*O*-methyl-nucleoside methyltransferases of vaccinia virus *in vitro* (194). Cycloleucine has also been shown to inhibit VSV replication in baby hamster kidney and Chinese hamster ovary cells (35, 243a), indicating that methylation may play an important role in viral mRNA synthesis *in vivo*. However, inhibition of methylation (AdoHcy) *in vitro* has virtually no effect on VSV mRNA transcription, although it affects the polyadenylation process. poly(A) tails 2,000 bases long (approximately 10 times the normal length) were added to VSV mRNA's when mRNA synthesis was carried out *in vitro* in the presence of AdoHcy (207).

CONCLUSIONS

The appearance of the cap structure in eucaryotic mRNA's is clearly an important step in the evolution of living organisms. Presumably, biosynthesis of guanylyltransferase and mRNA (7-guanine)methyltransferase and the appearance of these enzymes in the nuclei of primitive eucaryotic cells marked the onset of the first production of capped mRNA's. Ribose methylation and internal methylations of mRNA are late evolutionary events, the roles of which are still not clear. The methylation of the blocking guanosine base at position 7 is also an important process because it renders the cap structure polar, thus allowing interaction with charged macromolecules. In addition, the triphosphate bridge confers a freedom of movement of γ mG for similar interactions. Moreover, the structure appears to be resistant to the 5'-exonuclease action and thus may increase the half-life of the message. The involvement of the cap in mRNA translation has been documented adequately. More extensive studies on the CBP with regard to its structure and function will certainly help to elucidate the involvement of the cap in ribosome recognition. The roles of ribose methylation, N^6 mA^m, and internal N^6 mA in mRNA's are not well understood. Possibly, they are involved in fine tuning during initiation complex formation (121, 175), mRNA processing, and transport (23). It is not clear whether γ mG has any role in the transcription of the embryonic genome during preimplantation development of mammalian embryos. There is one report (275a) describing the appearance of γ mG in the RNA of mouse one-cell embryos 3 h after fertilization. This in contrast to the echinoderms, in which maternal mRNA's are capped and their translation after fertilization does not require attachment of γ mG to the 5' terminus (69, 109, 224). The mechanism of capping in the viral systems has been well elucidated, and the enzymes involved in the capping reactions have now been purified from both viral and eucaryotic sources. On the other hand, an understanding of capping mechanisms in cellular mRNA is still not clear. With the continued use of more refined *in vitro* nuclear systems, more light should be shed on this process in the near future. Elucidation of this reaction will be of great help in understanding the more general process of mRNA biosynthesis in eucaryotic cells in general. Future experiments will clearly be directed toward a more precise understanding of the function of the cap in eucaryotic gene expression.

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